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Yersinia pestis pH 6 Antigen: Genetic, Biochemical, and Virulence Characterization of a Protein Involved in the Pathogenesis of Bubonic Plague

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We studied a protein antigen, designated pH 6 Ag, that has the same regulation of expression as the previously described *Yersinia pestis* pH 6 Ag. Monospecific antiserum to this antigen recognized several proteins, ranging from 15 to over 75 kilodaltons (kDa), which were strongly expressed when *Y. pestis* was cultivated at 37°C and pH 6 but were expressed weakly, if at all, at 37°C and pH 8 and at 26°C. The antigen appeared to be composed of aggregates of a 15-kDa subunit. *Escherichia coli* minicell analysis and Western blotting (immunoblotting) of minicell extracts containing the cloned pH 6 Ag locus revealed that a 1.7-kilobase-pair (kb) region of *Y. pestis* chromosomal DNA produced 16- and 15-kDa immunoreactive proteins. We used transposon mutagenesis of the pH 6 Ag-coding region to demonstrate that the 16- and 15-kDa polypeptides were produced by the same cistron. The pH 6 Ag structural gene, *psaA*, was located within a 0.5-kb region of DNA. A Tn*10lacZ* transposon insertion 1.2 kb upstream of the *psaA* locus but outside the *psaA* transcriptional unit caused decreased expression of pH 6 Ag in both *E. coli* and *Y. pestis* and defined the *psaE* locus necessary for maximum pH 6 Ag expression. This locus itself was not regulated by temperature or pH. However, *psaA* remained responsive to both of these environmental signals in a *Y. pestis psaE* mutant. Mutation of either *psaE* or *psaA* resulted in at least a 100-fold reduction in the intravenous 50% lethal dose of *Y. pestis* in mice. Accordingly, pH 6 Ag is involved in the pathogenesis of bubonic plague.

Yersinia pestis is a facultative intracellular parasite and the causative agent of bubonic plague. The organism can survive and multiply in phagolysosomes of macrophages, which provide an environment in which other organisms are rapidly killed (8, 9, 34, 35). This pathogen also can grow extracellularly in blood and tissue spaces, despite the presence there of monocytes and polymorphonuclear neutrophils with oxidative killing mechanisms. Little is known about the gene products necessary for survival and multiplication of *Y. pestis* in these special environments.

In 1961, Ben-Efraim et al. (1) reported a new antigen, pH 6 Ag, synthesized by *Y. pestis* at temperatures similar to that of the mammalian body and at pH values similar to those in macrophage phagolysosomes or abscesses. This antigen was expressed by yersiniae growing in vivo. Further, *Y. pestis* cells strongly expressing pH 6 Ag were more rapidly fatal to mice than were yersiniae not strongly expressing this antigen, suggesting that pH 6 Ag may be involved in the pathogenesis of bubonic plague. Later, purified pH 6 Ag was found to be cytotoxic to cultured macrophages (2), raising the possibility that pH 6 Ag may be involved in the interaction between *Y. pestis* and host phagocytes. With this thought in mind, we used the previously described extraction procedure (2) to identify an antigen, designated pH 6 Ag, and characterized some of its genetic, biochemical, and pathogenic properties.

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MATERIALS AND METHODS

Bacterial strains, plasmids, and bacteriophage. The bacterial strains, plasmids, and bacteriophage used in this study are listed in Table 1.

Media and growth conditions. Routine culture of *Y. pestis* at 26°C was in brain heart infusion (BHI) (Difco Laboratories, Detroit, Mich.) broth or BHI agar. For determination of pH 6 Ag expression under various growth conditions, *Y. pestis* was grown in supplemented BHI (SBHI) containing 0.5% yeast extract (Difco) and adjusted to either pH 8 or pH 6 with NaOH or HCl, respectively. After autoclaving, 2.5 mM $CaCl_2$ and 0.2% (wt/vol) xylose were added. Cultivation of *Y. pestis* for the determination of beta-galactosidase activity was in the defined medium of Straley and Bowmer (32). In all cases in which pH 6 Ag expression was to be determined, the pH of the culture supernatant was determined immediately after the cells were harvested.

Escherichia coli strains were grown in Luria broth (LB) or on LB agar plates (28). *E. coli* cultures to be infected with bacteriophage lambda were grown in tryptone broth as described previously (28).

For selection of antibiotic resistance phenotypes of both *Y. pestis* and *E. coli*, the following antibiotic concentrations were used: chloramphenicol (CM), 25 μg/ml; ampicillin (AP), 100 μg/ml; kanamycin (KM), 25 μg/ml; tetracycline (TC), 25 μg/ml; streptomycin (SM), 100 μg/ml; and nalidixic acid (NL), 40 μg/ml.

DNA techniques and genomic library construction. Plasmid DNA was isolated by the method of Birnboim and Doly (3). Rapid screening of plasmid size was as previously described (13). Whole-cell DNA was isolated by the method of Marmur (19). Agarose and polyacrylamide gel electrophoresis of DNA restriction fragments were as described previously (18). Restriction enzymes, T4 DNA ligase, and calf intestine

TABLE 1. Bacterial strains, plasmids and bacteriophage

Strain, plasmid, or bacteriophage	Relevant comments[a]	Source or reference
Strain		
Y. pestis		
KIM5	Pgm$^-$	R. R. Brubaker
KIM5-3005	Spontaneous Nal^r mutant of KIM5	This study
KIM5-3001	Spontaneous Str^r mutant of KIM5	Laboratory stock
KIM5-3001.1	Pgm$^-$ *psaA3*::m-Tn*3*Cm; resolved *psaA* mutant of pDG25 heterozygous merodiploid in KIM5-3001	This study
KIM5-3005.1	Pgm$^-$ *psaE1*::Tn*10lacZ*; resolved *psaE* mutant of pPSN1 heterozygous merodiploid in KIM5-3005	This study
E. coli K-12		
HB101	*pro leu thi lacY hsd-20 endA recA rpsL20 ara-14 galK2 xyl-5 mtl-1 supE44*	4
M2141	*minA minB* Δ(*pro-lac*) Str^r	F. Neidhardt
NS2114Sm	*recA rpsL* (λ *cre*)	26, 27
POI1734	*ara*::(Mu cts)*3* Δ(*pro-lac*) *XIII rpsL*, with Mu dI1734	7
RDP146	*recA rpsE* Δ(*pro-lac*)	26, 27
Sm10(λ *pir*)	*thi leu thr tonA lacY supE recA* [(RP4-2-Tc::Mu)] (λ *pir*)	V. Miller; 12, 23, 29
Sm10(λ *pir*)Sm	Spontaneous Str^r mutant of Sm10(λ *pir*)	This study
Plasmid		
pDG1	pH 6 Ag-positive cosmid clone in pHC79 vector; Ap^r	This study
pDG4	9-kb *Cla*I fragment containing pH 6 Ag locus from pDG1 cloned into vector pJM703.1 *Cla*I site; Ap^r	This study
pDG6	9-kb *Cla*I fragment containing pH 6 Ag locus cloned from pDG1 into vector pHSS6 *Cla*I site; Km^r	This study
pDG9	3-kb *Kpn*I-to-*Bam*HI fragment containing pH 6 Ag locus cloned from pDG6 into vector pIC20R; Ap^r	This study
pDG10	2.6-kb *Eco*RI-to-*Bam*HI fragment containing pH 6 Ag locus cloned from pDG9 into pHSS6 vector; Km^r	This study
pDG11	1.7-kb *Eco*RI-to-*Bam*HI fragment containing *psaA* locus cloned into pHSS6; Km^r	This study
pDG24	*psaA3*::m-Tn*3*Cm (m-Tn*3*Cm insert 3 [Fig. 6]) in pDG10; Km^r Cm^r	This study
pDG25	*psaA3*::m-Tn*3*Cm cloned from pDG24 on a single *Not*I fragment into *Not*I-cleaved pVM703.1; Cm^r Ap^r	This study
pHC79	Cosmid cloning vector; Ap^r Tc^r	10
pHSS6	Cloning vector for m-Tn*3* mutagenesis; Km^r	26, 27
pIC20R	General cloning vector; *lacZ*α; Ap^r	20
pJM703.1	Suicide vector which contains the origin of replication of R6K and the Mob region of RP4; must be replicated and mobilized in Sm10(λ *pir*); Ap^r	23
pOX38::m-Tn*3*Ap	F plasmid derivative used to deliver m-Tn*3*Ap transposon; Ap^r	26, 27
pOX38::Tn*3*Cm	F plasmid derivative used to deliver m-Tn*3*Cm transposon; Cm^r	26, 27
pPSN1	*psaE1*::Tn*10lacZ* operon fusion in pDG4; Tc^r Ap^r	This study
pTCA	pACYC184 derivative that expresses Tn*3* transposase constitutively; Tc^r	27
pVM703.1	Derivative of pJM703.1 suicide vector containing a single *Not*I restriction site; Ap^r	V. Miller
Bacteriophage λ 1045	Delivery vector for Tn*10lacZ* element 14; λ *b221*, *cI857* P_{am}*80*	21

[a] Nal^r, Nalidixic acid resistant; Str^r, streptomycin resistant; Pgm$^-$, mutant which is defective in iron acquisition and which does not product a pigmented colony on Congo red agar.

phosphatase were obtained from Bethesda Research Laboratories (Gaithersburg, Md.) and used according to the specifications of the manufacturer. DNA restriction fragments fractionated on agarose gels were transferred to nitrocellulose by the method of Southern (31). DNA probes were prepared with the Dupont, NEN Research Products (Boston, Mass.) nick translation kit. DNA restriction fragments were purified by electroelution (18). Filter hybridization and posthybridization washes were as described by Silhavy et al. (28). Washed and dried filters were autoradiographed at −70°C with X-Omat AR film (Eastman Kodak Co., Rochester, N.Y.) and E. I. du Pont de Nemours & Co. (Wilmington, Del.) Cronex Lightning-Plus screens.

A total genomic library of *Y. pestis* DNA was constructed. Cellular DNA was partially digested with *Bam*HI (18), and fragments were isolated and ligated to *Bam*HI-cut, dephosphorylated pHC79, as previously described (14). Cosmids were packaged by using the Packagene (Promega Corp., Madison, Wis.) lambda DNA packaging kit according to the instructions of the manufacturer.

E. coli was transformed with plasmid DNA by the method of Brown et al. (6).

Preparation of pH 6 Ag-specific antiserum. Crude pH 6 Ag extracts were prepared from *Y. pestis* KIM5 cultures by potassium thiocyanate (KSCN) extraction of whole cells grown at 37°C and pH 6 in SBHI, as described elsewhere (2). Each of three 7-lb (ca. 3-kg) female New Zealand White rabbits was injected with crude KSCN extracts both intramuscularly and intraperitoneally. The injected material was a 1:1 emulsion of 500 μg of pH 6 Ag KSCN extract in phosphate-buffered saline (150 mM NaCl, 1.7 mM KH_2PO_4, 5 mM Na_2PO_4 [pH 7.4]) with Freund complete adjuvant (Calbiochem-Behring, La Jolla, Calif.). This procedure was repeated at 2-week intervals with Freund incomplete adju-

vant (Calbiochem) for a total of five sets of injections per rabbit. Antibody production was monitored by the Ouchterlony immunodiffusion reaction. Positive sera were pooled and brought to 50% saturation with ammonium sulfate. Precipitated antibody was pelleted by centrifugation, suspended in phosphate-buffered saline, and dialyzed against phosphate-buffered saline overnight at 4°C.

Anti-pH 6 Ag antibody (Ab) (50 ml) was adsorbed with lyophilized *Y. pestis* KIM5 which had been grown to an A_{620} of 1.0 in a 10-liter volume of SBHI at pH 8 and 37°C in a Magnaferm fermentor (New Brunswick Scientific Co., Inc., Edison, N.J.). Following slow shaking at 37°C for 1 h, the antibody preparation was clarified by centrifugation at 4°C.

PAGE, Western blotting (immunoblotting), and minicell analysis. Proteins to be analyzed by one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (16) were usually boiled in electrophoresis sample buffer, stored at −20°C, and electrophoresed without further boiling. However, for one experiment (see Fig. 2), all sample preparation was done just prior to electrophoresis. Two-dimensional nonequilibrium pH gradient gel electrophoresis in the first dimension and SDS-PAGE in the second dimension were as previously described (24). Protein concentration was determined by the method of Bradford (5).

For Western blot analysis, proteins were transferred to nitrocellulose by the method of Towbin et al. (36) as described previously (17), by using anti-pH 6 Ag Ab at a dilution of 1/750. The molecular weights of the reactive proteins were determined by cotransfer of ^{14}C-methylated standards (Amersham Corp., Arlington Heights, Ill.) and autoradiography of the dried membranes.

E. coli minicells were isolated and labeled with [^{35}S]methionine, as described elsewhere (15), and analyzed by SDS-PAGE. The gels were impregnated with En^{3}Hance (Dupont, NEN Research Products), dried, and exposed on X-Omat AR film (Kodak) at −70°C.

Immunological detection of pH 6 Ag expression by bacterial colonies. *E. coli* colonies were grown on nitrocellulose membranes on LB agar at 30°C. The membranes were placed in a chloroform vapor tank for 30 min, washed three times for 10 min in Western buffer containing 40 μg of lysozyme and 1 μg of DNase I per ml, and reacted with anti-pH 6 Ag Ab, as done in Western blotting.

Transposon mutagenesis. Recombinant plasmids containing the pH 6 Ag coding region were mutagenized with the minitransposon Tn*3* (m-Tn*3*), as described by Seifert et al. (26, 27). Mutagenesis with the minitransposon Mu dI1734 (m-Mu dI1734) was essentially as described previously (7). Briefly, pDG9 was transformed into m-Mu dI1734-containing *E. coli* POI1734. After induction of transposition and packaging of Mu, the resulting bacteriophage lysate was used to transduce *E. coli* M2141 to Ap^r Km^r. Transductants were selected on MacConkey lactose agar (Difco) plates at 30°C. Plasmids from Lac$^+$ colonies were analyzed for the point of m-Mu dI1734 insertion by digestion with *Hin*dIII and *Bam*HI.

For transposon Tn*10lacZ* mutagenesis of pDG4, *E. coli* Sm10(λ *pir*) containing pDG4 was infected with λ 1045 as described previously (28). The cells were washed to remove unadsorbed phage, diluted with LB containing TC, and shaken at 37°C for 4 h. Transposon inserts in pDG4 were then selected by conjugally transferring plasmid DNA into a Sm^r host, with simultaneous selection for vector and transposon antibiotic resistances. Approximately 5×10^8 donor cells were washed once with LB and mixed with recipient *E. coli* Sm10(λ *pir*)Sm cells at a ratio of 2:1 (donor to recipient). The mixture was then collected onto a 0.22-μm-pore-size filter (Millipore Corp., Bedford, Mass.) and incubated on LB agar at 37°C for 1 h. Transconjugants were selected on LB agar containing AP, SM, KM, and TC and screened for pH 6 Ag production by the colony blot procedure described above.

Recombination mutagenesis of *Y. pestis*. The suicide vectors pJM703.1 and pVM703.1 were used to introduce pH 6 Ag transposon mutations into the *Y. pestis* chromosome, as described by Miller and Mekalanos (12, 23). These plasmids require that factors necessary for replication and mobilization be supplied in *trans*. *E. coli* Sm10(λ *pir*) contains a λ *pir* lysogen which supplies the needed replication protein and a Mu lysogen which provides the required mobilization functions. However, in a noncomplementing host such as *Y. pestis*, these plasmids are unable to replicate. Antibiotic resistance conferred by the plasmid can be retained only by homologous recombination between cloned sequences on the chimeric plasmid and the recipient DNA. Accordingly, Sm10(λ *pir*)Sm containing pPSN1 was mated with *Y. pestis* KIM5-3005. In the case of pDG25, Sm10(λ *pir*) was mated with *Y. pestis* KIM5-3001. In both cases, filter matings were performed at a ratio of 1 donor per 10 recipients on SBHI overnight at 37°C. The next morning, growth from the filters was suspended in BHI. Heterozygous merodiploids were selected on NL-TC-AP (pPSN1) or SM-CM-AP (pDG25) tryptose blood agar (Difco). A single colony was grown at 30°C in BHI in the absence of AP selection and then plated onto tryptose blood agar CM-SM (pDG25) or TC-NL (pPSN1) plates. After 48 h of incubation at 30°C, the colonies were replicaplated onto like media containing AP. Resolution of the merodiploids occurred at frequencies of ca. 1 of 500 and 1 of 20,000 colonies screened for pPSN1 and pDG25, respectively.

Virulence testing of mice. Retro-orbital injection and 50% lethal dose (LD_{50}) determination of the *psaA Y. pestis* mutant for BALB/c mice were as previously described (33). An extra mouse injected with the highest dose was sacrificed 24 h postinjection. Organisms recovered on tryptose blood agar from its liver and spleen were judged to be the same as the injected *Y. pestis* mutant by colony morphology, antibiotic resistance profile, and plasmid profile.

RESULTS

Expression of pH 6 Ag by *Y. pestis*. Previously, Ben-Efraim et al. (1) described an antigen that was produced by *Y. pestis* only when cultivated at temperatures above 36°C and pH values below 6.7. We made KSCN extracts from *Y. pestis* KIM5 grown under various temperature and pH combinations. Extracts that had been made from cells grown at 37°C and pH 6 revealed specific immunoreactivity on Western blots (Fig. 1). We could detect pH 6 Ag when as little as 0.75 μg of protein from KSCN extracts from cells grown at 37°C and pH 6 were analyzed by Western blot. The newly expressed polypeptides had molecular masses ranging from 15 to greater than 75 kilodaltons (kDa). Furthermore, these proteins appeared to increase in size by increments of approximately 15 kDa, suggesting that the serologically reactive species might be composed of aggregates of a 15-kDa subunit. Thus, it appeared that our antibody was specific for a *Y. pestis* antigen with regulation of expression, as described for pH 6 Ag, and we have tentatively assigned it the pH 6 Ag designation of Ben-Efraim et al.

Characterization of pH 6 Ag subunit structure. Three experiments indicated that pH 6 Ag is made of aggregates of

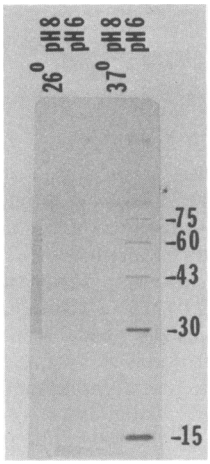

FIG. 1. Western blot of KSCN extracts prepared from *Y. pestis* KIM5 grown under various conditions of temperature and pH. Approximately 30 μg of protein from each sample was separated by SDS-PAGE (12.5% [wt/vol] acrylamide) and transferred to nitrocellulose. The Western blot was probed with anti-pH 6 Ag Ab, as described in Materials and Methods. Sizes of proteins, in kilodaltons, are indicated at right.

the 15-kDa protein rather than a mixture of distinct polypeptides. First, a sample of crude KSCN pH 6 Ag extract was mixed with an equal volume of 2× SDS-PAGE sample buffer. A sample was then removed, and the rest was boiled for 1 or 5 min. The sample that was not boiled revealed no 15-kDa proteins but contained a greater proportion of the 30-kDa and larger proteins compared with the boiled samples (Fig. 2). In contrast, boiling for only 1 min liberated the 15-kDa subunit and decreased the reactive species in the higher-molecular-weight range, eliminating the species at the

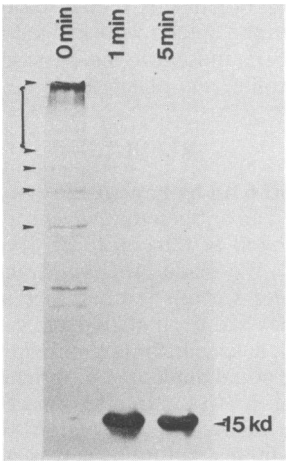

FIG. 2. Western blot of KSCN extracts prepared with or without boiling from *Y. pestis* KIM5 grown at 37°C and pH 6. Lane 0min, loaded directly without boiling of the sample; lane 1min, boiled for 1 min; lane 5min, boiled for 5 min. Approximately 40 μg of total protein was loaded in each lane. The arrows at the left indicate the positions of the high-molecular-weight aggregates of pH 6 Ag which dissociated into the 15-kDa subunit after the sample was boiled. The position of the 15-kDa (kd) subunit is indicated at the right. Faint reactivity of the higher-molecular-weight species was still present in the samples boiled for 1 and 5 min but is not visible in this photograph.

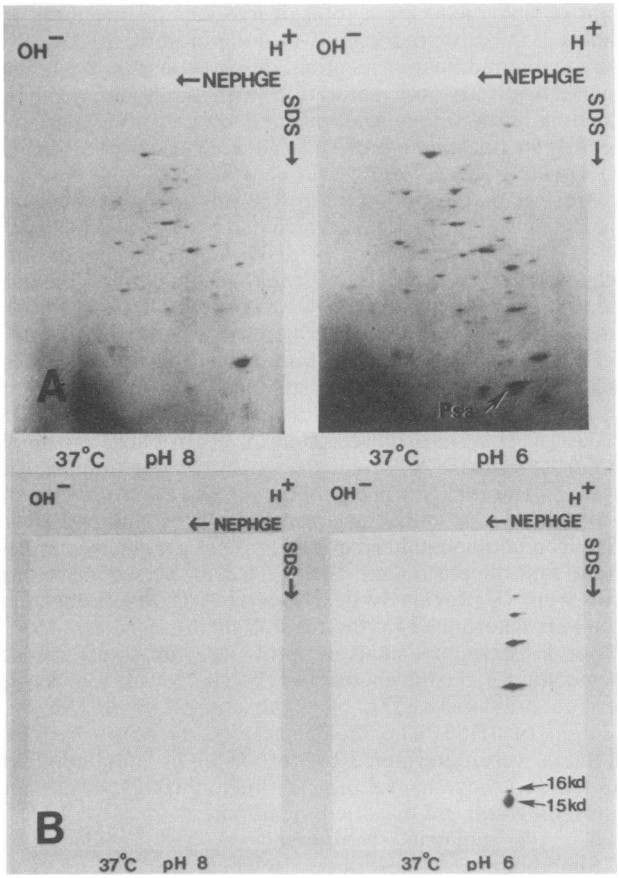

FIG. 3. Two-dimensional nonequilibrium pH gradient gel electrophoresis of KSCN extracts from *Y. pestis* KIM5 grown at 37°C and pH 6 or pH 8. (A) Coomassie blue-stained gel of nonequilibrium pH gradient gel electrophoresis-separated KSCN extracts. The samples were prepared from *Y. pestis* KIM5 cultivated as indicated in the figure. The position of the monomeric subunit of pH 6 Ag is indicated with the arrow labeled Psa. The dimeric and trimeric forms of pH 6 Ag can also be seen in this photograph. (B) Western blot of *Y. pestis* KSCN extracts separated by nonequilibrium pH gradient gel electrophoresis. The gels were loaded and run similarly to the ones shown in panel A. The reactivity associated with pH 6 Ag can be seen in the panel on the right. The positions (←) of the 16- and 15-kDa (kd) proteins are indicated. In both panels, ca. 40 μg of protein was electrophoresed in the first dimension.

very top of the gel altogether. (As shown in Fig. 1, freezing the boiled sample prior to electrophoresis permitted some reaggregation to occur.)

Second, we used a method of determining immunological cross-reactivity developed by Smith and Fisher (30). Crude pH 6 Ag extract was transferred to nitrocellulose and reacted with rabbit anti-pH 6 Ag Ab. A portion of the blot was removed and developed to visualize the various forms of pH 6 Ag. The developed strip was then aligned with the rest of the membrane, and sections from the undeveloped nitrocellulose were removed. Antibody bound to these strips was eluted and then used to probe Western blots of crude pH 6 Ag KSCN extracts. Using this procedure, we found that the antibodies eluted from the 30-, 43-, 60-, and 75-kDa bands all reacted with the 15-kDa polypeptide (data not shown). Thus, the 30-kDa and larger reactive proteins immunologically cross-reacted with the small 15-kDa subunit of pH 6 Ag.

Third, we subjected pH 6 Ag KSCN extracts to two-

dimensional gel electrophoresis (Fig. 3). When the gels were stained with Coomassie blue (Bio-Rad Laboratories, Richmond, Calif.), a low-molecular-weight acidic protein was present in extracts from cells incubated at 37°C and pH 6 but not in extracts from cells incubated at 37°C and pH 8 (Fig. 3A). When similar gels were subjected to Western blotting, a series of reactive proteins was detected, with masses ranging from ca. 15 to 75 kDa (Fig. 3B). These reactive polypeptides were seen only in the extracts made from *Y. pestis* cells grown at 37°C and pH 6. Also, all the reactive proteins were of one isoelectric form, further suggesting that pH 6 Ag is a homopolymer of the 15-kDa subunit and is not composed of heterogeneous species of proteins. However, another protein just above the 15-kDa subunit was visualized in this two-dimensional Western blot (Fig. 3B). To determine if the lack of detection of the 16-kDa protein on one-dimensional SDS-PAGE Western blots was a quantitative problem, we blotted various amounts of pH 6 Ag KSCN extracts. We found that more than 40 μg of extract was required for detection of the 16-kDa protein by one-dimensional electrophoresis.

Cloning pH 6 Ag in *E. coli*. We constructed a cosmid library in *E. coli* HB101 and screened 1,000 cosmid clones representing two genomic equivalents (14) for reactivity with our pH 6 Ag-specific antibody (see Materials and Methods). Nine positive clones were identified, purified, and characterized for their plasmid DNA content. Each clone had common 5.0-, 7.8-, and 9.6-kilobase-pair (kb) *Bam*HI fragments, suggesting that a single region of the *Y. pestis* chromosome encodes pH 6 Ag. Crude whole-cell extracts made from the clones and subjected to Western blotting revealed aggregates which comigrated with the reactive polypeptides of the *Y. pestis* positive control. However, pH 6 Ag expression was not regulated by either growth temperature or pH in *E. coli*. One of these cosmid clones containing 42 kb of *Y. pestis* DNA was chosen for further study and designated pDG1. When KSCN extracts were made from *E. coli* HB101 harboring pDG1, we found that pH 6 Ag was present, although in lesser amounts than in the extracts made from *Y. pestis* (data not shown).

From the original 50-kb pDG1 cosmid, we subcloned into the vector pHSS6 a 9-kb *Cla*I fragment which encoded a product that reacted with the anti-pH 6 Ag Ab (Fig. 4). Restriction maps of this fragment and two further subclones are shown in Fig. 4. All of the subclones produced the 16- and 15-kDa proteins in *E. coli* minicells (Fig. 4). However, the ratio of 16-kDa protein to 15-kDa protein was reversed in pDG6 and pDG10. When the 0.9-kb *Eco*RI fragment was removed from pDG10 to generate the pDG11 subclone, expression of both the 16- and 15-kDa proteins decreased. Western blots of minicell extracts made from pDG6, pDG10, and pDG11 all reacted with the anti-pH 6 Ag Ab at both the 16- and the 15-kDa bands (data not shown).

Transposon mutagenesis. We mutagenized pDG6 with m-Tn*3*Ap as described by Siefert *et al.* (26, 27). After mutagenesis, we patched colonies to nitrocellulose and determined their reactions with anti-pH 6 Ag Ab. After screening 450 colonies by this procedure, we obtained seven isolates that did not appear to react with anti-pH 6 Ag Ab on colony blots. The pH 6 Ag m-Tn*3*Ap insertions in all of these negative clones were located by restriction mapping and found to be between 4.0 and 5.5 kb (Fig. 5A). However, one of these m-Tn*3*Ap insertion mutants was found to produce a small amount of the 16- and 15-kDa proteins when *E. coli* minicells were examined (Fig. 5B and C, lanes 6). This insertion was mapped to within 50 base pairs of the *Bam*HI

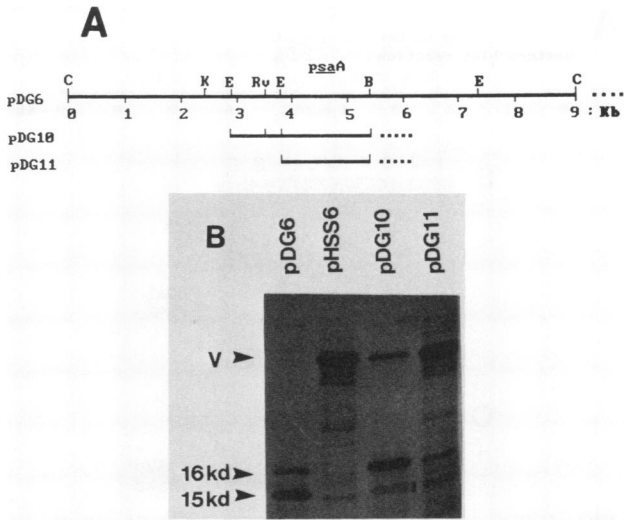

FIG. 4. *E. coli* minicell analysis of the pH 6 Ag-coding region. (A) Restriction map of the *Y. pestis* 9-kb *Cla*I fragment containing the pH 6 Ag-coding region and the maps of two subclones. ——, *Y. pestis* sequences contained in each plasmid; ····, vector sequences. The 1.7-kb *Eco*RI-to-*Bam*HI fragment containing the structural gene for pH 6 Ag is labeled (psaA) above the map. Restriction site abbreviations: C, *Cla*I; K, *Kpn*I; E, *Eco*RI; Rv, *Eco*RV; B, *Bam*HI. (B) Autoradiogram of ^{35}S-labeled plasmid proteins produced in *E. coli* minicells. Extracts were separated by SDS-PAGE (15% [wt/vol] acrylamide) and processed as described in Materials and Methods. Each lane is labeled with the name of the plasmid used. V, Position of the vector pHSS6-encoded neomycin phosphotransferase. The *Y. pestis*-specific 16- and 15-kDa proteins are indicated. Each lane represents ca. 150,000 cpm of ^{35}S-labeled extract.

restriction site (Fig. 5A). The other m-Tn*3*Ap insertions were mapped within 0.5 kb and did not produce either the 16- or the 15-kDa protein (Fig. 5). Transposon m-Tn*3*Ap insertions 4 and 3 (Fig. 5, lanes 4 and 3, respectively) revealed polypeptides in minicells that did not appear in the other transposon mutants. These specific proteins are probably truncated versions of pH 6 Ag (PsaA), and their molecular weights along with the locations of the m-Tn*3*Ap insertions allowed us to assign the *psaA* locus as shown in Fig. 5A.

We isolated five Lac$^+$ m-Mu dI1734 insertions in pDG9 (Fig. 6). Subclone pDG9 is a *Kpn*I-to-*Bam*HI fragment obtained from pDG6 (Fig. 5) and contains ca. 0.3 kb more *Y. pestis* DNA than pDG10 (Fig. 6). All five of the m-Mu dI1734 insertions were oriented in the same direction, as determined by *Bam*HI and *Hin*dIII restriction digests (Fig. 6). The direction of transcription is from left to right, as shown in Fig. 6. Three of the m-Mu dI1734 insertion mutants (Fig. 6, insertions a through c) did not synthesize either the 16- or 15-kDa protein. Two of the mutants (Fig. 6, insertions d and e) produced both of these immunoreactive proteins. Furthermore, m-Mu dI1734 insertions c and d are separated by only 0.1 kb (Fig. 6), yet c produced neither the 16- nor the 15-kDa protein, and insertion d produced both. Accordingly, these proteins can not be produced by separate cistrons, and therefore are likely synthesized by the same locus.

We isolated 60 m-Tn*3*Cm insertions in pDG10 and determined their locations by restriction digestion with *Bam*HI and double digestion with *Bam*HI and *Eco*RI. Five of these transposon insertions spaced at various places along the cloned sequence were chosen for further characterization. One of these mutants (Fig. 6, insertion 5) produced both the 16- and the 15-kDa proteins. The other four m-Tn*3*Cm

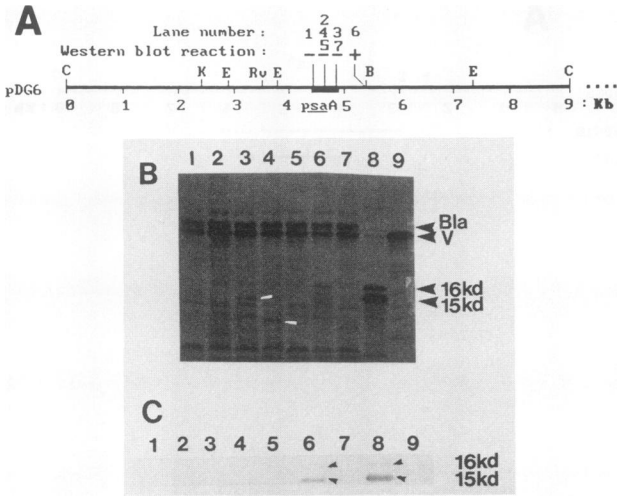

FIG. 5. Transposon m-Tn*3*Ap insertions in pDG6. (A) Restriction site cleavage map of pDG6 and location of m-Tn*3*Ap transposon insertions in or near the pH 6 Ag structural gene. Positive (+) and negative (−) Western blot reactions of extracts from the insertion mutants are indicated. The numbers above the position of the transposon insertion indicate the lane numbers for SDS-PAGE of the *E. coli* minicell extracts prepared from the mutants (B) or for Western blots of the same samples (C). The mutants in lanes 2, 4, and 5 and lanes 3 and 7 appeared to be in the same location when analyzed at the level of restriction digestion. The thick bar represents the location of *psaA*. Restriction site symbols are as defined in the legend to Fig. 4. (B) Autoradiogram obtained after SDS-PAGE (15% [wt/vol] acrylamide) of *E. coli* minicell extracts prepared from the transposon mutants or controls. Lanes 1 through 7, Mutants 1 through 7 (as labeled above the restriction map in panel A); lanes 8 and 9, ^{35}S-labeled plasmid proteins obtained from *E. coli* M2141 harboring pDG6 and pHSS6, respectively. The white arrows indicate the positions of the 14.5-kDa (lane 3) and 13-kDa (lane 4) proteins which may be the truncated products of *psaA* caused by the transposon insertions. The locations of the 16- and 15-kDa proteins in panels B and C are indicated to the right of the photograph. Protein abbreviations: Bla, β-lactamase; V, neomycin phosphotransferase. (C) Western blot of minicell extracts after SDS-PAGE as described for panel B. The lanes are numbered as in panel B. Note that the m-Tn*3*Ap mutant shown in lane 6 produced both 16- and 15-kDa species; however, these proteins are barely visible in the autoradiogram shown in panel B. In both panels B and C, 150,000 cpm of minicell extract was loaded in each lane.

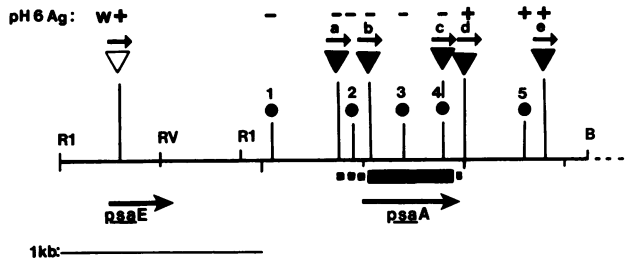

FIG. 6. Positions of transposon insertions in pDG10. Western blot reactions are indicated above the restriction map for the mutant having an insertion at that location. Symbols: +, positive reaction; −, negative reaction; w+, weak reaction; ●, m-Tn*3*Cm; ▼, m-Mu dI1734; ▽, Tn*10lacZ*. The m-Tn*3* mutants were assigned numbers (1 through 5) and the m-Mu insertions were assigned letters (a through e) as indicated. The arrows indicate the direction of transcription as determined by restriction mapping of the plasmids containing transposons which generate operon fusions. The solid line (from R1 to B) indicates the 2.6-kb region of *Y. pestis* DNA. The m-Mu dI1734 mutants were obtained by mutagenesis of pDG9, which contains approximately 0.3 kb more *Y. pestis* DNA to the left of the first *Eco*RI site shown here. ----, pHSS6 vector sequences; ▬, pH 6 Ag-coding region. R1, *Eco*RI; RV, *Eco*RV; B, *Bam*HI.

mutants were all negative for these proteins when analyzed by Western blotting. The m-Tn*3*Cm insertion designated no. 1 in Fig. 6 was located 0.5 kb upstream of *psaA*. This suggests that transcription of *psaA* initiates well before the pH 6 Ag structural gene.

A single Tn*10lacZ* insertion which mapped within the 0.9-kb *Eco*RI fragment was isolated (Fig. 6). This transposon insertion resulted in greatly decreased expression of *psaA*, as determined from Western blots of equivalent amounts of crude *E. coli* extracts. However, a small amount of PsaA was detected; therefore, the Tn*10lacZ* insertion did not abolish the transcription of *psaA*. Accordingly, this Tn*10lacZ* mutant defines a separate transcriptional unit (designated *psaE*) that exerts an effect on *psaA* expression. The direction of transcription of *psaE* was determined to be from left to right, as shown in Fig. 6. Transcription direction was determined by restriction mapping and β-galactosidase activity.

Isolation of pH 6 Ag mutants in *Y. pestis*. We used the suicide plasmid recombination mutagenesis scheme of Miller and Mekalanos (23) to deliver pH 6 Ag mutations to *Y. pestis*. *E. coli* Sm10(λ *pir*) harboring pDG25 was mated with *Y. pestis* KIM5-3001. The pDG25 *psaA* mutant plasmid cannot replicate in *Y. pestis* (see Materials and Methods). Thus, Cm^r Ap^r Sm^r colonies can arise only through recombination at homologous regions between pDG25 and the genome of *Y. pestis* (Fig. 7, steps 1 and 2). The m-Tn*3*Cm insertion present in pDG25 (Fig. 6, insertion 3) had been mapped at the middle of the *psaA* locus. A heterozygous merodiploid mutant of *Y. pestis* with a genome structure similar to the one shown in Fig. 7, step 2, was cultured extensively in the absence of AP selection. This was accomplished by making 1/100 dilutions daily, for 7 days, in BHI containing CM and SM. On day 7, ca. 1 in 20,000 cells in the population had become Ap^s while retaining Cm^r. The structure of this resolved mutant is depicted in Fig. 7, step 3. Southern hybridization with the 1.7-kb *Bam*HI-to-*Eco*RI fragment of pDG11 confirmed that no gross chromosomal rearrangements had occurred during the recombination mutagenesis of *Y. pestis* (data not shown). Western blots with three Cm^r Sm^r Ap^s PsaA$^-$ *Y. pestis* isolates confirmed that there was no production of pH 6 Ag (data not shown).

We repeated the recombination mutagenesis described above and in Fig. 7, with Sm10(λ *pir*) harboring pPSN1 as the donor and *Y. pestis* KIM5-3005 as the recipient. However, after growth overnight and a single 1/100 dilution in the absence of AP selection, we recovered resolved (Ap^s) mutants at a frequency of 1 per 500 colonies screened. The increased flanking homology present on pPSN1 may have enhanced the recombination frequency between directly repeated sequences in the *Y. pestis* heterozygote (analogous to step 2 in Fig. 7). Western blots of the *psaE* mutant generated in this way revealed less than 1/10 the wild-type level of PsaA. Thus, we confirmed our finding in *E. coli* with the *psaE*::Tn*10lacZ* mutation in *Y. pestis*. β-Galactosidase activities under various growth conditions were determined for *Y. pestis* KIM5-3005.1 (*psaE*::Tn*10lacZ*) (22). Regardless of the temperature or pH at which the *psaE*::Tn*10lacZ* fusion mutant was cultivated, it produced 1,000 to 2,000 Miller units (22) of β-galactosidase. In contrast, the parent *Y. pestis* KIM5-3005 negative-control strain produced only 10

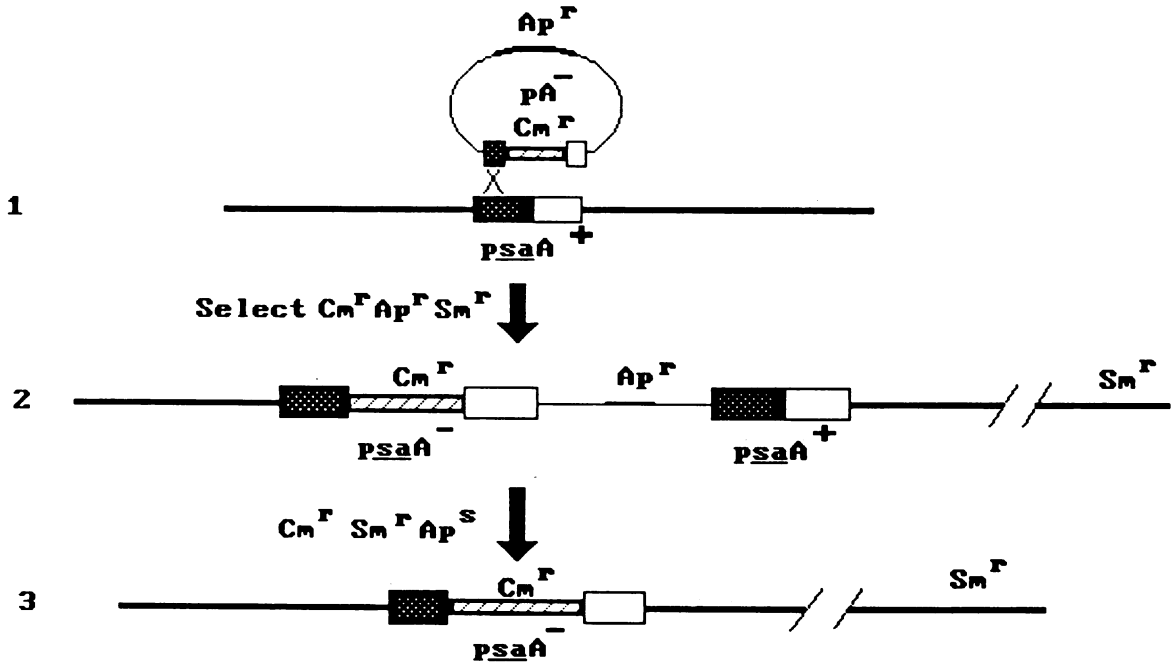

FIG. 7. Generation of the PsaA⁻ mutant of *Y. pestis* by recombination. Homologous sequences in the cloned *psa* DNA of the suicide plasmid pDG25 and in the *Y. pestis* genome recombine (step 1) in a single crossover to generate a heterozygous merodiploid (step 2). After many generations in the absence of selection for Ap^r encoded by the suicide vector but with continued Cm^r and Sm^r selective pressure, the directly repeated regions of *psaA* and *psaA*$^+$ recombine to yield a stable PsaA⁻ mutant (step 3). ——, Vector sequences; ▬, *Y. pestis* DNA; ▩ and □, homologous regions of the *psaA* locus; ▨, m-Tn*3*Cm sequences. The slightly thickened area of the plasmid sequences represents the Ap^r determinant present on the pVM703.1 vector.

Miller units of β-galactosidase. Thus, *psaE* expression does not respond to growth temperature or pH.

Virulence testing. We tested the effect that loss of pH 6 Ag had on the virulence of *Y. pestis* by retro-orbital injection of BALB/c mice with various doses of the *psaA*::m-Tn*3*Cm mutant *Y. pestis* KIM5-3001.1. The 50% lethal dose of this mutant was 9×10^3. In comparison, the 50% lethal dose of the parent *Y. pestis* strain was 4.2×10^1. Thus, the loss of PsaA had increased the 50% lethal dose of *Y. pestis* over 200-fold by the intravenous route of infection. We also tested the virulence of the *Y. pestis* KIM5-3005.1 (*psaE*::Tn*10lacZ*) mutant. Sets of five mice were injected with doses of 10^3 or 10^6 bacteria per mouse. None of the five mice injected with 10^3 *Y. pestis* KIM5-3005.1 died. In contrast, all of the mice injected with the 10^3 dose of the parent strain died within 6 days after inoculation. Both mutant and parent strains killed all the mice which were injected with the 10^6 dose. Thus, it appears that loss of the *psaE* product and decreased expression of PsaA are sufficient to cause reduced virulence of *Y. pestis*.

DISCUSSION

We have been successful in demonstrating that the *Y. pestis* pH 6 Ag is involved in the pathogenesis of bubonic plague. Using pH 6 Ag-specific antibody, we showed that the antigen formed aggregates which were partially stable to treatment with SDS and β-mercaptoethanol. Several lines of evidence presented here suggest that the aggregates of pH 6 Ag are a homopolymer of the 15-kDa protein. First, the aggregates are composed of species having a single acidic isoelectric point, as indicated by two-dimensional gel electrophoresis (Fig. 3). Second, immunological cross-reactivity demonstrated that antibodies which bound to the higher-molecular-weight forms of pH 6 Ag also recognized the 15-kDa subunit. Third, when antigen preparations were heated in the presence of SDS and β-mercaptoethanol, the higher-molecular-weight species gave rise to the 15-kDa subunit (Fig. 2). Fourth, the higher-molecular-weight species of pH 6 Ag increased in increments of approximately 15 kDa (Fig. 1). Fifth, transposon mutagenesis of the pH 6 Ag coding region revealed that aggregate production, i.e., antigen production, was completely abolished or completely present for insertions separated by only 0.1 kb (Fig. 6, m-Mu dI1734 insertions c and d). These facts taken together demonstrate that the immunologically reactive polypeptides which constitute pH 6 Ag are not distinct proteins but are oligomers of the 15-kDa subunit protein.

We detected a 16-kDa protein produced by *Y. pestis* and *E. coli* harboring the cloned pH 6 Ag which reacted to pH 6 Ag-specific antibody. Transposon mutagenesis of the pH 6 Ag locus revealed that the 16-kDa protein and the 15-kDa protein were produced by the same cistron (Fig. 6). The simplest explanation for this result is that the 16-kDa polypeptide is a precursor of the 15-kDa subunit of pH 6 Ag. The removal of 1 kDa could represent the cleavage of a secretion signal peptide. This possibility is supported by the fact that pH 6 Ag is extracted with membrane-chaotropic agents such as KSCN and thus appears to be membrane associated. However, confirmation of this possibility will await DNA sequence analysis and cell fractionation experiments.

We found pH 6 Ag (PsaA) to be encoded within a 1.7-kb *Eco*RI-to-*Bam*HI region of *Y. pestis* chromosomal DNA (Fig. 4). The *psaE* locus located on a 0.9-kb *Eco*RI fragment directly upstream of *psaA* was found to lie in a distinct

transcriptional unit and to be necessary for the maximal expression of pH 6 Ag. These conclusions are made from several results. First, when the 0.9-kb *Eco*RI fragment containing *psaE* was deleted from pDG10 to generate pDG11, PsaA was still expressed in *E. coli* minicells, but at dramatically decreased levels for both the 16- and 15-kDa proteins (Fig. 4). Second, the Tn*10lacZ* transposon insertion which defines *psaE* is located in the center of this fragment, away from the junction with the *psaA*-containing region. When the *psaE*::Tn*10lacZ* mutation was recombined into *Y. pestis*, the fusion mutant still produced PsaA, but at a barely detectable level. Although *psaA* expression responds to growth temperature and pH, the *psaE* locus is not regulated at the transcriptional level by either of these two environmental stimuli. Furthermore, *psaA* remains regulated by both temperature and pH in the PsaE$^-$ mutant. Accordingly, PsaE is necessary for the maximal expression of *psaA;* however, an as-yet-undefined locus (loci) is responsible for thermal and acidic regulation of the antigen structural gene.

Loss of pH 6 Ag resulted in a >200-fold increase in the 50% lethal dose of the *psaA* mutant compared with that of the parent *Y. pestis* strain. These data corroborate the previous finding that *Y. pestis* strains which express pH 6 Ag are more rapidly fatal to mice (1). Previously, pH 6 Ag had been shown to have cytotoxic activity toward mouse peritoneal macrophages, to cause inflammation, and to result in the agglutination of erythrocytes (2). However, it is not known whether these properties underlie its function in vivo. *Y. pestis* has an additional cytotoxic component encoded by the low-Ca^{2+}-response virulence plasmid pCD1 (11). Thus, *Y. pestis* produces multiple gene products which could be involved in the interaction of the organism with host phagocytes.

During an infection, *Y. pestis* inhabits two environments which are known to be ca. 37°C and acidic and which therefore could be sites of expression of pH 6 Ag: the phagolysosome of macrophages, in which *Y. pestis* can survive and grow (33–35), and the extracellular environment in abscesses such as buboes and lesions formed in liver and spleen (33, 37). These are special niches, with properties not present in the general circulatory system or in tissues. Such special environments act as cues for virulence gene expression by *Y. pestis*. A previously described example of this is the low-Ca^{2+}-response virulence property, by which yersiniae respond to the combined cues of mammalian body temperature and the presence or absence of Ca^{2+} to regulate expression of a set of virulence genes. The phagolysosome of macrophages is a host microenvironment that provides these cues and elicits low-Ca^{2+}-response gene expression (25). Thus, pH 6 Ag enlarges the application of this concept for *Y. pestis*. This pathogen senses multiple properties of the microenvironments created by its own host-pathogen interactions and responds by the appropriate expression of virulence properties.

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